AGRICULTURAL MATERIALS

Reversed-Phase Ion-Pair Liquid Chromatographic Determination of Chlorophacinone and Diphacinone in Steam-Rolled Oat Baits and Steam-Rolled Oat/Wax Baits

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A reversed-phase ion-pair liquid chromatographic (LC) method was developed for analysis of steam-rolled oat (SRO) baits fortified with either chlorophacinone or diphacinone. Baits were prepared with and without paraffin wax. Chlorophacinone or diphacinone was extracted from wax-free SRO baits with 5 mM tetrabutylammonium phosphate methanolic ion-pairing solution. Wax baits were initially extracted with petroleum ether and then cleaned up by liquid extraction into methanolic ion-pairing solution containing 20% water. SRO extracts were analyzed with reversed-phase ion-pair LC. Chlorophacinone and diphacinone were quantified by UV absorption at 325 nm. Recoveries from SRO fortified with chlorophacinone at 25 and 150 μg/g were 90.7 and 90.8%, respectively, whereas for diphacinone at the same levels, recoveries were 93.5 and 92.3%, respectively. Recoveries from wax baits fortified at 25 and 75 μg/g chlorophacinone were 98.5 and 100%, respectively, whereas for diphacinone at the same levels, recoveries were 93.6 and 98.0%, respectively. Method limits of detection for chlorophacinone and diphacinone in SRO baits were estimated to be 1.0 and 0.76 μg/g, respectively. Method limits of detection for chlorophacinone and diphacinone in wax baits were estimated to be 4.2 and 2.8 μg/g, respectively.

Diphacinone (2-(diphenylacetyl)-1H-indene-1,3(2H)-dione) and chlorophacinone (2-[(4-chlorophenyl)phenylacetyl]-1H-indene-1,3(2H)-dione) are registered anticoagulant rodenticides commonly used for controlling rats at dosage levels below those required by most other anticoagulant rodenticides. These anticoagulants also are effective in control of rangeland rodents such as Valley pocket gophers (*Thomomys bottae*), Belding ground squirrels (*Spermophilus beldingi*), and California ground squirrels (*Spermophilus beecheyi*). Pocket gophers and ground squirrels are vectors for diseases such as bubonic plague. These rangeland rodents can also reduce vegetation by 20 to 30%, which results in less plant material for livestock grazing. Additionally, the combination of grazing by pocket gophers, ground squirrels, and livestock can lead to severe soil erosion. Damage to earthen irrigation ditches and dams has been observed in areas where pocket gopher and ground squirrel populations have become excessive (1, 2). Control methods for ground squirrels and pocket gophers include exclusion, shooting, trapping, flooding, and use of acute toxicants including acute anticoagulants and fumigants (3). Steam-rolled oat (SRO) baits fortified at 50 and 100 μg/g chlorophacinone or diphacinone are used in California grasslands to control rodent populations. Wax baits fortified at 50 μg/g chlorophacinone or diphacinone are used in wetter regions of California to control rodent populations. Chlorophacinone- and diphacinone-fortified baits are formulated by small independent companies with limited quality control resources. To assist with registration of these formulations for protection of agriculture and public health, we developed practical methodology to verify the concentration of the active ingredients in these baits.

Several methods have been developed for analysis of indanediones in baits, formulations, and tissues. Each of these methods has some advantages and disadvantages. Gas chromatographic methods with derivatization (4) are sensitive and selective but suffer from low recoveries and lengthy preparation time. Spectrophotometric methods (5) have been used for baits and formulations, but they are not selective when multiresidue samples are being assayed. Thin-layer chromatographic (6–8) methods are not suited for determination of low levels of residues in complex matrixes or for accurate quantitation. Reversed-phase high performance liquid chromatography (LC) methods (9–13) provide the required sensitivity but often produce poor chromatographic resolution. Reversed-phase ion-pair LC (14–17) has adequate sensitivity, and selectivity, but column deterioration is often a problem.

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Reversed-phase ion-pair LC was evaluated as the most appropriate method of analysis for our purposes because of the potentially good chromatographic resolution; column deterioration can be controlled reasonably well with column washing if it is done regularly (18). This method was simple and rapid. This method was validated for ground SRO containing 25 to 150 μg/g chlorophacinone or diphacinone and for wax baits containing 25 to 75 μg/g chlorophacinone or diphacinone.

Experimental

Apparatus

The LC system consisted of a Hewlett-Packard 1090 liquid chromatograph (Palo Alto, CA) operated at 35°C. A Hewlett-Packard 1050 variable-wavelength detector at 325 nm was used to detect chlorophacinone and diphacinone. The analytical wavelength of 325 nm was chosen over the more sensitive wavelength of 285 nm because the occurrence of a late-eluting peak is minimized when 325 nm is used as the analytical wavelength. A pneumatically controlled injector valve automatically injected 25 μL portions into the chromatograph. Analytes were separated on a 25 × 0.46 cm id stainless steel analytical column packed with 5 μm Keystone ODS/H (Bellefonte, PA) with a flow rate of 1.0 mL/min. To prolong column lifetime, a 1.5 × 0.46 cm id Keystone ODS/H guard column was used. The mobile phase was prepared by mixing aqueous and methanolic solutions of 5 mM tetrabutylammonium dihydrogen phosphate (20 + 80, v/v) and adjusting pH to 7.5 with 4N phosphoric acid. The mobile phase was degassed by sparging with helium. At the end of each set of analyses, the column was washed with methanol–water (1 + 1, v/v) for 40 min.

Operating conditions were adjusted occasionally to maintain optimum response and reproducibility. With these conditions, retention times of diphacinone and chlorophacinone were ca 4.5 and 6.5 min (Figure 1).

Reagents

Petroleum ether, ethyl acetate, and methanol were LC grade (Fischer Scientific, Denver, CO). Deionized water was purified with a Milli-Q water purification system (Millipore, Bedford, MA). Concentrated phosphoric acid (Fischer Scientific) was used to make 4N phosphoric acid in water.

Tetrabutylammonium dihydrogen phosphate (97%) from Aldrich (Milwaukee, WI) was used to prepare the 5 mM solution in methanol. A commercially prepared tetrabutylammonium dihydrogen phosphate ion-pairing reagent with buffer (potassium dihydrogen phosphate) was purchased from Alltech, Inc. (Deerfield, IL) and used to make the 5 mM solution in water.

Indanedione Standards

Chlorophacinone (98.9%) was obtained from LiphaTech (Milwaukee, WI), and diphacinone (99.3%) was obtained from Hacco, Inc. (Madison, WI). All concentrated and fortification standard solutions were prepared as separate solutions of chlorophacinone or diphacinone and not combined standards.

(a) *Concentrated stock standards and fortification standards (1000 μg/mL chlorophacinone or diphacinone).*—Prepared by first drying the technical-grade compounds for 4 h at 110°C and then dissolving 10.000 mg analyte in ethyl acetate in a 10 mL volumetric flask and diluting to volume with ethyl acetate.

(b) *Fortification standards (10 000 μg/mL chlorophacinone or diphacinone).*—Prepared by dissolving previously dried 100.00 mg diphacinone or chlorophacinone in ethyl acetate in a 10 mL volumetric flask and diluting to volume with ethyl acetate.

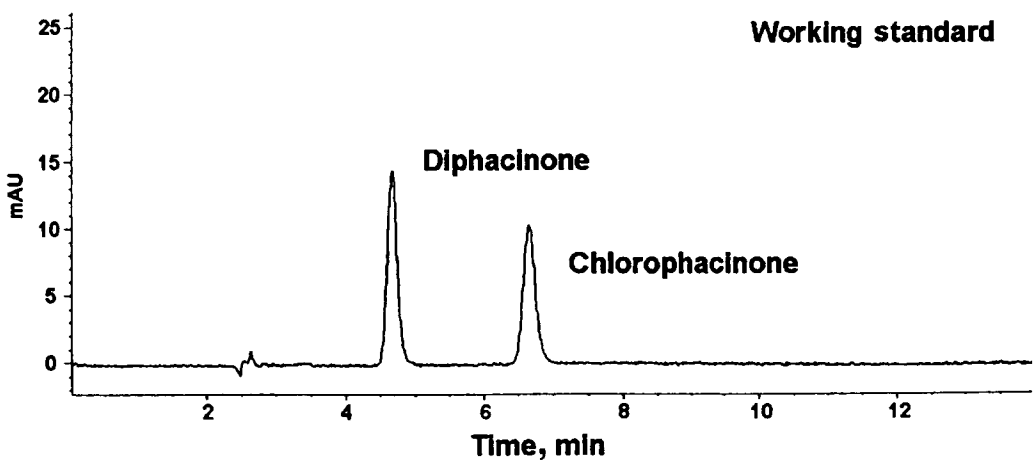

Figure 1. Chromatograms of a 1.0 μg/mL chlorophacinone and 1.0 μg/mL diphacinone working standard with ultraviolet detection at 325 nm.

(c) *Working standards ranging in concentration from 0.8 to 22.0 μg/mL.*—Prepared by diluting stock solutions with mobile phase. All standard solutions were stored in a refrigerator at 5°C.

Fortification of Control SRO Baits

Control baits consisted of SROs, Alcolec S as a binder, and Dupont Oil Blue A as a marker dye. Control baits were ground to a fine powder with an electric coffee mill (Krups, Type 203B) and stored in a sealed container. The method was validated at 2 levels of chlorophacinone and diphacinone: 25 and 150 μg/g. Each 1.00–1.10 g portion of ground SRO bait was fortified with one analyte by adding 25.0 μL of the 1000 μg/mL or 15.0 μL of the 10 000 μg/mL fortification standard solution in ethyl acetate to produce the appropriate fortification level. Tubes containing fortified SRO controls were then placed under a stream of nitrogen to evaporate the ethyl acetate from the fortification standard.

Fortification of Control Wax Baits

Control wax baits consisted of SROs, paraffin wax, Alcolec S as a binder, and Dupont Oil Blue A as a marker dye. Control baits were ground with a hand-powered grinding mill (Fischer Scientific) into pieces of wax and oats no larger than a quarter of an inch in diameter. This was then ground into a fine powder with an electric coffee mill and stored in a sealed container. The method was validated at 2 levels of chlorophacinone and diphacinone: 25 and 75 μg/g. Each 1.00–1.10 g portion of ground wax bait was fortified with either chlorophacinone or diphacinone by adding 25.0 or 75.0 μL of the 1000 μg/mL standard solution in ethyl acetate to produce the appropriate fortification level. Tubes containing fortified controls were then placed in a warm water bath at 70°C to melt the wax and encapsulate the analytes as in the actual baits, as well as to evaporate the ethyl acetate from the fortification standard.

Sample Extraction

(a) *Extraction of SRO baits.*—Ground SRO samples were weighed accurately in 1.00 g portions into a 50 mL screw-cap polypropylene tube. Then 10.0 mL methanolic ion-pairing solution was pipetted into the sample tube. The tube was shaken on a Vortex mixer for 10 s and then shaken horizontally with a mechanical shaker (Eberbach Corp., Ann Arbor, MI) at high speed for 15 min. Sample tubes were then sonicated for 3 consecutive 15 min periods, with the tubes shaken by hand for a few seconds between each period. Sample tubes were centrifuged at ca 2500 rpm for 5 min. A portion of the extract was filtered with a 0.45 μm Teflon syringe filter into a 2 mL sample vial, the vial was capped, and the sample was analyzed by LC.

(b) *Extraction of wax baits.*—Ground wax samples were weighed accurately in 1.00 g portions into a 50 mL screw-cap polypropylene tube. Then 20 mL petroleum ether was poured into the sample tube. The tube was shaken on a Vortex mixer for 10 s and then shaken horizontally with a mechanical shaker (Eberbach Corp.) at high speed for 15 min. Sample tubes were then sonicated in a beaker for 3 consecutive 15 min periods, with the tubes shaken by hand for a few seconds between each period. Then 20 mL methanolic ion-pairing solution with 20% water was pipetted into the sample tube. The tube was shaken on a Vortex mixer, shaken horizontally, and centrifuged as was done with the wax-free SRO baits. The petroleum ether layer (top layer) was removed from the tube, and the methanolic layer was transferred to a 25 mL volumetric flask. The sample tube was washed with two 1.5 mL portions of the methanolic solution, and the washes were transferred to the 25 mL volumetric flask, diluted to volume with the methanolic solution, and mixed well. The methanolic extract was filtered with a 0.45 μm Teflon syringe filter into a 2 mL sample vial. The vial was capped, and the sample analyzed by LC.

Results and Discussion

Response Linearity

Two sets of 5 calibration standard solutions were prepared, ranging in concentration from 0.8 to 22 μg/mL. Each standard solution was injected 2 times, and a linear regression was performed on the data set. The regression statistics are shown in Table 1.

A linear relationship existed between analyte chromatographic peak response and analyte concentration, and the response was directly proportional to concentration over the range of interest. Single-point calibrations were valid over the range of standard solution concentrations.

Extraction

Diphacinone and chlorophacinone residues were extracted from ground samples with methanolic ion-pairing solution. For wax baits, petroleum ether was used to dissolve the wax to allow encapsulated analytes to be extracted with the methanolic ion-pairing:water solution.

Table 1. Regression statistics

Compound	r^2	Slope	y intercept
Peak response vs concentration			
Diphacinone	0.9997	71.99	0.494
Chlorophacinone	0.9996	63.95	0.155
Log (peak response) vs log (concentration)			
Diphacinone	0.9991	1.001	
Chlorophacinone	1.017	0.9995	

Recoveries

Mean recoveries ± standard deviations of chlorophacinone from SRO baits (n = 7 for all validation levels) at the 25 and 150 μg/g levels were 90.7 ± 2.5% and 90.8 ± 2.9%, respectively (Table 2). Mean recoveries of diphacinone ± standard deviations from SRO baits at the 25 and 150 μg/g levels were 93.5 ± 2.9% and 92.3 ± 3.3%, respectively (Table 2). Mean recoveries of chlorophacinone from SRO/wax baits (n = 7 for

Table 2. Fortification of control SRO baits and recoveries

Sample	Concentration of stock solution, μg/mL	Volume, mL	Sample weight, g	Theoretical concentration, μg/g	Observed concentration, μg/g	Receovery, %
			Diphacinone, 150 μg/g			
1	9970	0.0150	1.03	145	129	89.0
2	9970	0.0150	1.02	147	141	95.9
3	9970	0.0150	1.00	150	136	90.7
4	9970	0.0150	1.00	150	144	96.0
5	9970	0.0150	1.01	148	141	95.3
6	9970	0.0150	1.01	148	131	88.5
7	9970	0.0150	1.01	148	134	90.5
					Mean	92.3
					SD	3.3
					CV	3.6%
			Diphacinone, 25 μg/g			
1	997	0.0250	1.04	24.0	22.0	91.7
2	997	0.0250	1.01	24.7	23.3	94.3
3	997	0.0250	1.05	23.7	22.3	94.1
4	997	0.0250	1.01	24.7	24.0	97.2
5	997	0.0250	1.03	24.2	23.3	96.3
6	997	0.0250	1.01	24.7	22.6	91.5
7	997	0.0250	1.01	24.7	22.0	89.1
					Mean	93.5
					SD	2.9
					CV	3.1%
			Chlorophacinone, 150 μg/g			
1	9975	0.0150	1.03	145	130	89.7
2	9975	0.0150	1.02	147	136	92.5
3	9975	0.0150	1.00	150	130	86.7
4	9975	0.0150	1.00	150	143	95.3
5	9975	0.0150	1.01	148	137	92.6
6	9975	0.0150	1.01	148	134	90.5
7	9975	0.0150	1.01	148	131	88.5
					Mean	90.8
					SD	2.9
					CV	3.2%
			Chlorophacinone, 25 μg/g			
1	998	0.0250	1.04	24.0	21.5	89.6
2	998	0.0250	1.01	24.7	22.6	91.5
3	998	0.0250	1.05	23.8	22.0	92.4
4	998	0.0250	1.01	24.7	23.4	94.7
5	998	0.0250	1.03	24.2	21.8	90.1
6	998	0.0250	1.01	24.7	22.1	89.5
7	998	0.0250	1.01	24.7	21.5	87.0
					Mean	90.7
					SD	2.5
					CV	2.8%

Table 3. Fortification of control wax baits and recoveries

Sample	Concentration of stock solution, μg/mL	Volume, mL	Sample weight, g	Theoretical concentration, μg/g	Observed concentration, μg/g	Recovery, %
			Diphacinone, 75 μg/g			
1	1027	0.0760	1.06	73.6	71.1	96.6
2	1027	0.0760	1.06	73.6	72.2	98.1
3	1027	0.0760	1.12	69.7	68.5	98.3
4	1027	0.0760	1.01	77.3	75.2	97.3
5	1027	0.0760	1.01	77.3	75.3	97.4
6	1027	0.0760	1.06	73.6	72.9	99.0
7	1027	0.0760	1.04	75.0	74.6	99.5
					Mean	98.0
					SD	1.0
					CV	1.0%
			Diphacinone, 25 μg/g			
1	1027	0.0250	1.03	24.9	23.1	92.8
2	1027	0.0250	1.05	24.5	22.3	91.0
3	1027	0.0250	1.02	25.2	22.9	90.9
4	1027	0.0250	1.02	25.2	23.6	93.7
5	1027	0.0250	1.01	25.4	23.5	92.5
6	1027	0.0250	1.07	24.0	23.7	98.8
7	1027	0.0250	1.02	25.2	24.1	95.6
					Mean	93.6
					SD	2.8
					CV	3.0%
			Chlorophacinone, 75 μg/g			
1	1024	0.0760	1.07	72.7	73.4	101
2	1024	0.0760	1.03	75.6	76.1	101
3	1024	0.0760	1.09	71.4	73.0	102
4	1024	0.0760	1.24	62.8	61.7	98.2
5	1024	0.0760	1.04	74.8	75.3	101
6	1024	0.0760	1.00	77.8	79.3	102
7	1024	0.0760	1.03	75.6	72.5	95.9
					Mean	100
					SD	2.3
					CV	2.3%
			Chlorophacinone, 25 μg/g			
1	1024	0.0250	1.01	25.3	24.2	95.7
2	997	0.0250	1.02	25.1	23.1	92.0
3	997	0.0250	1.03	24.9	25.5	102
4	997	0.0250	1.05	24.4	24.1	98.8
5	997	0.0250	1.00	25.6	25.9	101
6	997	0.0250	1.09	23.5	23.6	100
7	997	0.0250	1.02	25.1	25.1	100
					Mean	98.5
					SD	3.5
					CV	3.6%

all validation levels) at the 25 and 75 μg/g levels were 98.5 ± 3.5% and 100 ± 2.3%, respectively (Table 3). Mean recoveries of diphacinone from SRO/wax baits at the 25 and 75 μg/g levels were 93.6 ± 2.8% and 98.0 ± 1.0%, respectively (Table 3). Recovery data collected from quality control samples analyzed with actual samples, prepared by various independent contractors, are shown in Table 4. Representative control samples (all components except diphacinone and chlorophacinone) were treated according to the procedures in this method. Recoveries were not significantly different at the levels chosen, which bracket the target concentration. Chromatograms of a commercially prepared chlorophacinone- and diphacinone-fortified SRO and wax bait control samples are shown in Figures 2 and 3 for comparison. As can be seen in Figures 2A and 3A, no chromatographic responses were observed at, or very near, the retention time of chlorophacinone or diphacinone in all control samples. A late-eluting peak was observed that may cause problems in subsequent chromatograms. However this can be avoided by appropriately adjusting run time.

The active ingredient concentration in SRO bait is calculated as follows:

$$\text{Analyte, } \mu\text{g/g} = \frac{A_u}{A_{std}} \times C_{sta} \times \frac{10.00 \text{ mL}}{\text{sample wt (g)}}$$

where A_u = peak area of analyte in sample, A_{std} = peak area of analyte in standard, and C_{std} = concentration of standard (μg/mL).

The active ingredient concentration in wax bait is calculated as follows:

$$\text{Analyte, } \mu\text{g/g} = \frac{A_u}{A_{std}} \times C_{std} \times \frac{25.00 \text{ mL}}{\text{sample wt (g)}}$$

where A_u = peak area of analyte in sample. A_{std} = peak area of analyte in standard, and C_{std} = concentration of standard (μg/mL).

Method Limit of Detection

The method limit of detection (MLOD) was defined as the concentration of chlorophacinone or diphacinone required in the sample to generate a signal equal to 3 times the baseline noise (peak to peak) observed in the chromatogram of the control extract. The MLOD was estimated from the chromatographic response of the analyte in height for extracts of a control bait sample and a control bait sample fortified at 25 μg/g. Under the conditions specified in the method, MLODs for SRO bait were 1.0 μg/g for chlorophacinone and 0.76 μg/g for diphacinone. Under the conditions specified in the method, MLODs for wax bait were 4.2 μg/g for chlorophacinone and 2.8 μg/g for diphacinone.

Conclusions

These methods for analysis of chlorophacinone- or diphacinone-fortified SRO and wax baits are simple, precise, and accurate. They provide high daily sample throughput for both SRO bait (n = 30) and wax bait (n = 20). These methods will be used to support laboratory and field efficacy studies in hopes of registering formulations for rodent control and protection of agriculture and public health.

Acknowledgments

We are grateful to Hacco, Inc., and LiphaTech, Inc., for supplying diphacinone and chlorophacinone and to the California Department of Food and Agriculture for providing funding and control samples.

Table 4. Recoveries of quality control samples of fortified SRO and wax baits determined with actual samples

Analyte	Type of bait	Fortification, μg/g	Number of replicates[a]	Mean recovery, %	Standard deviation	Coefficient of variation, %
Diphacinone	SRO	100	6	94.7	4.9	5.2
Diphacinone	SRO	50	6	95.6	4.5	4.7
Diphacinone	Wax	50	6	101	1.3	1.3
Chlorophacinone	SRO	100	24	90.3	4.8	5.3
Chlorophacinone	SRO	50	12	91.6	7.0	7.6
Chlorophacinone	Wax	50	3	97.5	2.1	2.2

[a] Three quality control samples were assayed for each set of baits analyzed.

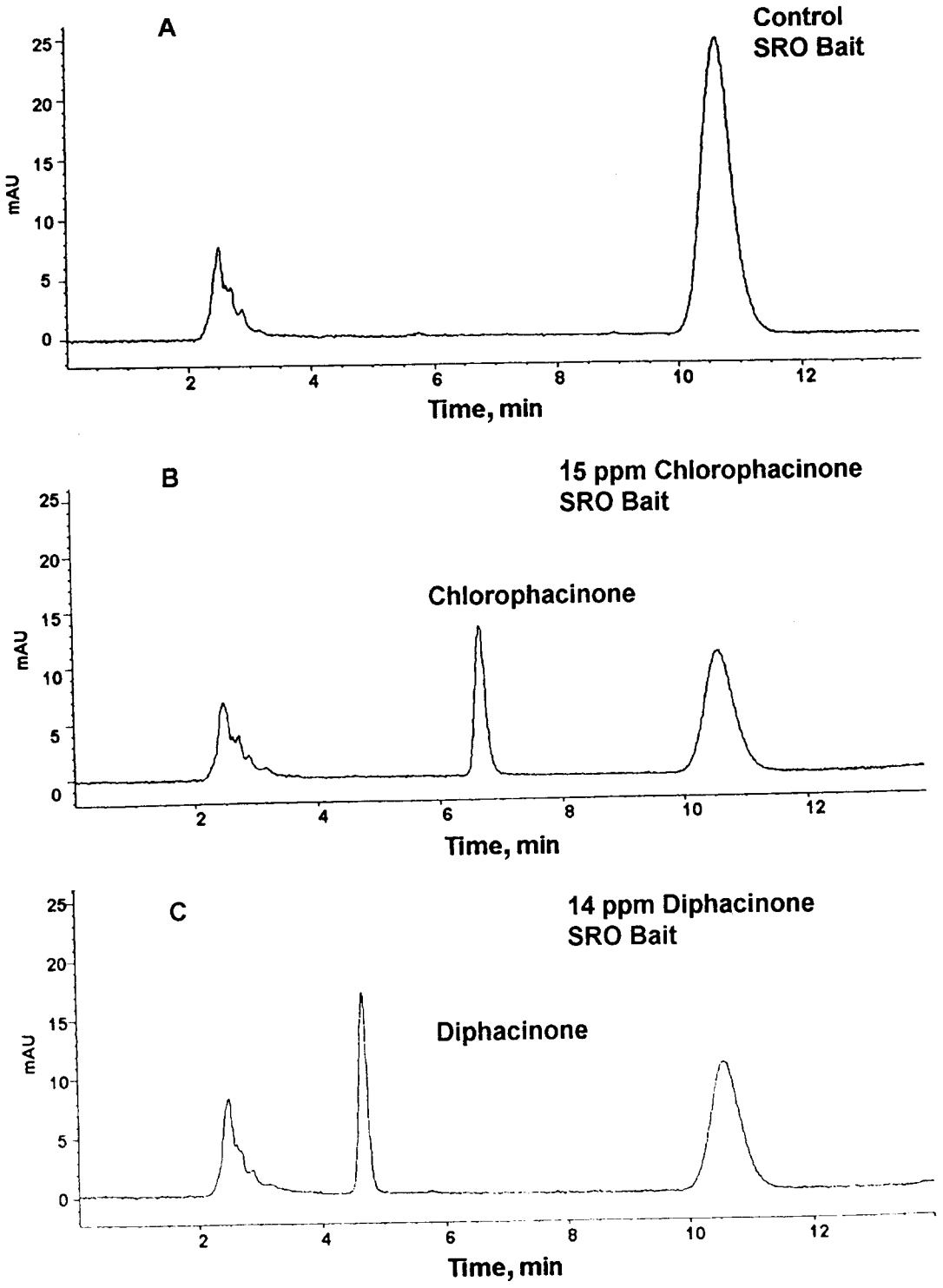

Figure 2. Chromatograms of (A) a blank SRO control sample extract, (B) a 15 μg/g SRO bait chlorophacinone sample extract, and (C) a 14 μg/g SRO bait diphacinone sample extract.

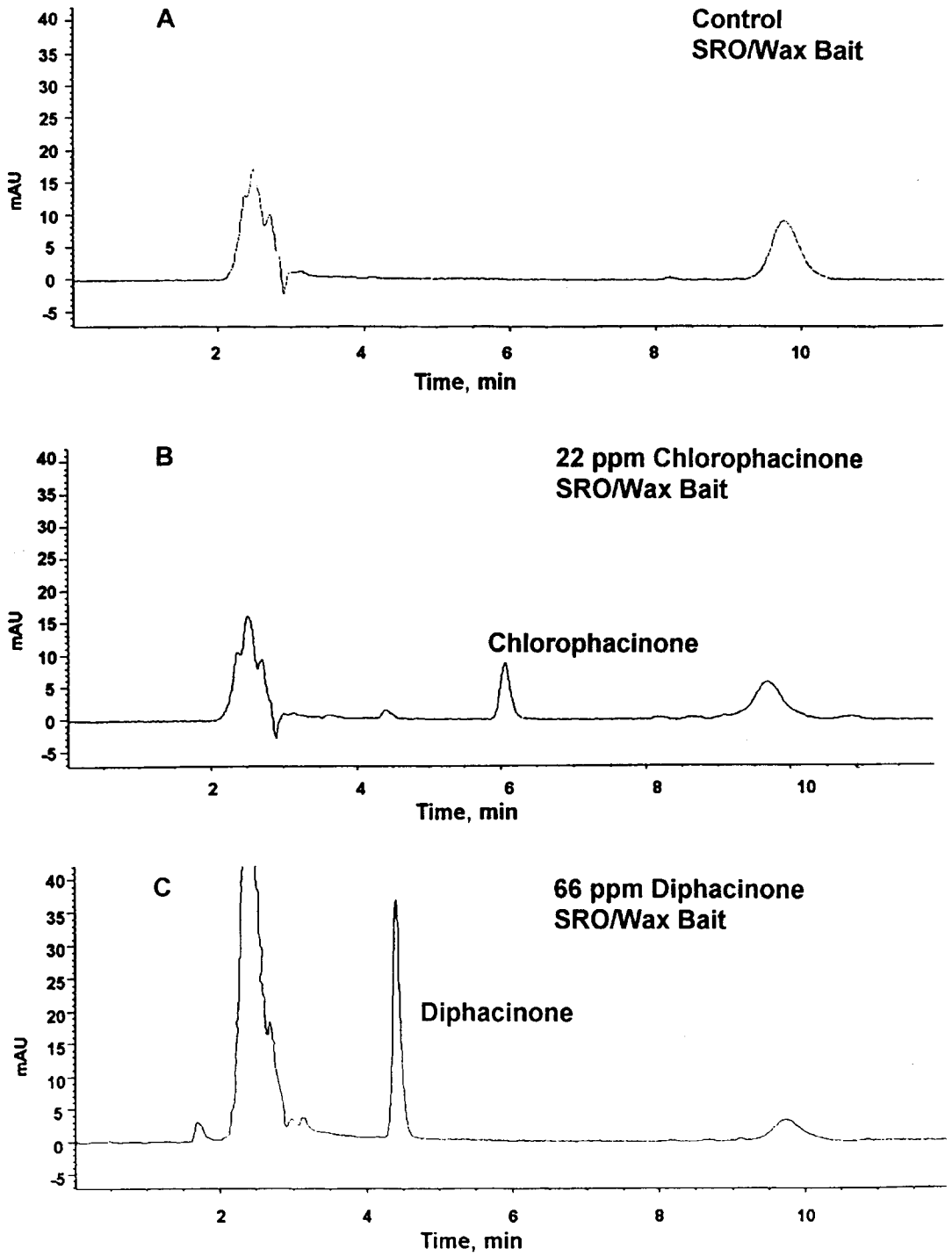

Figure 3. Chromatograms of (A) a blank SRO control sample extract, (B) a 22 μg/g SRO/wax bait chlorophacinone sample extract, and (C) a 66 μg/g SRO/wax bait diphacinone sample extract.

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